Seasonal dynamics of macroinvertebrates communities in alpine ponds

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

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Dissertação submetida à Faculdade de Ciências da Universidade do Porto, para a obtenção do grau de Mestre em Ecologia e Ambiente, da responsabilidade do Departamento de Biologia.
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Resumo

As lagoas alpinas são corpos de água naturais de pequenas dimensões e pouco profundos formados nas zonas alpinas, que se caracterizam por apresentarem, normalmente, condições pristinas. As condições extremas que ocorrem nestes ecossistemas aquáticos permitem a emergência de comunidades biológicas únicas com grande valor de conservação. Assim, é de extrema importância reunir informação acerca destes ecossistemas. De acordo com esta informação, o objetivo da presente tese prendeu-se com a caraterização sazonal da comunidade de macroinvertebrados bentónicos de lagoas alpinas em Portugal. Em Portugal continental, a zona alpina começa a partir dos 1700 metros de altitude, e apenas a Serra da Estrela apresenta lagoas com caraterísticas alpinas. Deste modo, foram selecionadas 5 lagoas alpinas para a realização deste estudo. A comunidade de macroinvertebrados bentónicos foi amostrada em três períodos distintos (junho, outubro e abril), e adicionalmente, em cada lagoa foram determinados parâmetros físico-químicos *in situ* e recolhidas amostras de água para posterior análise laboratorial (ex: concentração de clorofila *a*, nitratratos). Relativamente aos resultados obtidos para os parâmetros físico-químicos estes foram semelhantes aos registados para outros lagos e lagoas alpinas da Europa, com valores de pH ligeiramente acídicos e baixa condutividade. Na análise comparativa das lagoas, feita através de uma análise de componentes principais, foram observadas diferenças na caraterização química da água ao longo dos diferentes períodos amostrados. Nomeadamente, em outubro com baixos valores de oxigénio e elevada concentração nitratos. Variações sazonais foram observadas na comunidade de macroinvertebrados nas lagoas 3 e 10 com um decréscimo da abundância e riqueza específica em outubro. A análise de correspondência permitiu ainda descriminar diferenças na composição das comunidades de duas lagoas (4 e 12). A formação de uma base de dados de macroinvertebrados nestes ecossistemas é importante não apenas para registar as espécies ocorrentes, mas também poderá permitir uma deteção precoce de alterações significativas nestes ecossistemas.

Palavras-chave: Ecossistema aquático, lagoas de pouca profundidade, qualidade ecológica, Península Ibérica, Serra da Estrela, parâmetros físicos e químicos, índices bióticos – IBMWP, diversidade, riqueza taxonómica, equitabilidade.

Abstract

Alpine ponds are small and shallow natural waterbodies formed in alpine zones and are characterized by their usual pristine conditions. The extreme harsh conditions of this environment enable the emergence of unique communities with high conservational value. Therefore, it is extremely important to gather information about these ecosystems. In accordance with this information, the aim of this study was to do a seasonal characterization of the benthic macroinvertebrates of Portugal's alpine ponds. In continental Portugal, the alpine zone starts at 1700 meters a.s.l. and only Serra da Estrela has ponds with alpine characteristics. Thus, 5 alpine ponds were selected to conduct the present study. Benthic macroinvertebrates community was sampled in three distinct periods (June, October and April), and, additionally, *in situ* physical and chemical parameters were determined for each pond. Water samples were collected to perform further analysis (e.g.: chlorophyll a concentration, nitrates, among others). Regarding to the water physical and chemical data obtained, these were in accordance with other studied lakes and ponds in alpine zones in Europe, as slightly acidic pH values and low conductivity. In the comparative analysis of the ponds, made by a Principal Component Analysis, differences in water chemistry across the seasons were observed. Namely, October had low oxygen values and high nitrate concentration. Seasonal shifts in macroinvertebrates communities were also noticed for pond 3 and 10, with a decrease in abundance and taxonomic richness in October. A Correspondence Analysis allowed the discrimination of differences in communities' composition of two different ponds (4 and 12). The formation of a base dataset of macroinvertebrates in these ecosystems is important not only to record the occurring species but also to allow an early detention in macroinvertebrates communities shifts due to global warming.

Keywords: Aquatic ecosystem, shallow lakes, ecological quality, Iberian Peninsula, Serra da Estrela, physical and chemical parameters, biotic indexes – IBMWP, diversity, taxonomic richness, evenness.

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Introduction

Lake ecology

The scientific interest in water bodies started around mid-nineteenth-century, when Henry Thoreau made notes about Walden Pond, and with the description of Lake Geneva by Forel, in 1892, that defined Limnology as “the oceanography of lakes”. Later, in the 30’s, Edgardo Baldi described limnology as the study of the processes and their relations by which matter and energy varied within a lake. Limnology is not easy to define, being commonly accepted as the science of inland waterbodies (including lotic and lentic habitats, independently of the size and depth), their biological communities and the factors that affect them (Cole 1976).

Several factors can form depressions in the ground that are later filled with water, forming the currently named lakes. These factors are mainly tectonic, glacial or fluvial processes, but lakes can also be formed by landslides, volcanoes, meteor’s craters or even by the action of the wind (Bengtsson 2012a). Lake’s basic hydrology is dictated by water inflow and outflow, with precipitation and evaporation taking a major role in a lake’s hydrodynamics, and being highly influenced by climate and geological processes (Bengtsson 2012b). Some lakes have no outflow and are considered terminal lakes, also called *endorheic lakes*, and other lakes that have water outflow are referred as *spillover lakes* (Bengtsson 2012b). There are also lakes associated with the groundwater system in which most of the water inflow and outflow are associated with the groundwater flow (Bengtsson 2012b). It is also important to notice that lake’s life cycle, their basins, and their morphology are fundamentally dictated by geological processes. Their overall basins concave shape implies a gradual filling process by sediments that are pushed to the lake during its maturing process until their eventual obliteration (Cole 1976).

Solar radiation in lake’s surface accounts for the major input of thermal energy and it is important because water density is dependent of temperature. Temperature is the essential variable to the formation of thermal layers in deep waterbodies, promoting stratification. Stratification is the development of water layers in a lake with distinct abiotic characteristics, being that the top layers are generally high illuminated with high dissolved oxygen values, and consequently hotter than deeper layers (Bengtsson 2012c; Huttula 2012). Normally, the stratification process leads to the formation of three distinct layers. Epilimnion is the upper layer that is hotter than the rest of the lake; metalimnion, the mid layer, is where occurs an accentuated drop in water temperature, having a zone where temperature drops quicker that is called thermocline; and the deeper layer, hypolimnion, has equally cold temperature across the layer (Huttula 2012; D. W. Schindler 1991). In specific ecosystems with extreme winter conditions, it may occur inverse stratification. When this phenomenon occurs, the top layer is ice covered and is the lightest but coldest and the deeper layer is warmer. This type of stratification happens because the ice insulates the water from the atmosphere, thus maintaining the top layer cold while heat is released at the bottom by the sediments. (Bengtsson 2012c; Huttula 2012). Stratification can have consequences with ecological relevance. Metalimnion can isolate hypolimnion, stopping the last layer to exchange gases with the atmosphere. Moreover, this deep layer present low photosynthetic production, due to lack of solar radiation reaching it. Consequently, a depletion of oxygen in this layer was a problem for the biota, increasing due to the presence of decomposing bacteria that use the low quantity of oxygen that may be still in the layer quicker (Huttula 2012). However, this stratification may not be constant during the year. Stratification may vary from amictic lakes that are ice covered and never have a turnover to polymictic lakes that have turnovers several times a year (Bengtsson 2012c). Monomictic lakes have a turnover once a year, oligomictic have only very seldom turnovers and are generally in stormy periods. Dimictic lakes have two turnovers a year (generally in autumn and spring) and meromictic lakes, where hypolimnion is isolated, and the turnover occurs only in epilimnion and metalimnion (Huttula 2012). The smaller and shallower polymictic lakes may stratify and de-stratify several times in a year. This situation occurred due to strong winds that mix the water or may even never stratify due to constant water mixing. These lakes have only one layer with the characteristics of the epilimnion layer (Schindler 1991).

Lakes (water bodies) are generally classified by their trophic state, and that can be eutrophic or oligotrophic (terms first used by Weber in 1907 describing north German peat bogs), but many lakes may be stated in a transitional state, a mesotrophic state. Oligotrophic lakes are characterized by transparent blue or green waters with high values of dissolved oxygen but with low nutrient concentration and with low organic matter in the sediments. Eutrophic lakes tend to be less transparent, with water colors varying between yellow to brownish green, with oxygen depletion in summer but rich in nutrients and with high quantity of organic matter in the sediments (Table 1). There are two main factors which interaction dictates lakes trophic state; the morphology of the lake (dimensions of catchment area), and edaphic factor (properties of surrounding soils) as the geological factors and the climatic element (duration of the growing season, solar radiation, precipitation, the wind, evapotranspiration rates and temperature differences)

(Cole 1976). These factors allied with anthropogenic pollution may influence the inflow of nutrients and that can lead to eutrophication of the waterbody. In normal conditions, algal biomass and aquatic plants growth are restricted by the lack of a nutrient (normally phosphorous and nitrates), so that the growth rate of algae and aquatic plants is proportional to the nutrient supply rate. However, excessive amounts of nutrients (namely, phosphorous and nitrates) may reach a waterbody, which leads to an uncontrolled rise in algae and aquatic plants abundance that increases drastically the system's productivity. This process conduct to species loss and changes in communities structures, with an effect on the ecosystem services provisioned and consequent economic losses (Smith et al. 1998).

Table 1 - Basic differences between oligotrophic and eutrophic lakes. Adapted from Cole, (1976).

Oligotrophy	Eutrophy
Marked transparency	Limited transparency
Water poor in nutrients	Abundant nutrients in the water
High values of dissolved oxygen	Oxygen depleted in summer hypolimnion
Low primary production	High primary production

Physical and chemical factors are also a major importance in the dynamics of lakes, and are closely related to the geology and biology of these aquatic environments. Environmental variations and catchment proprieties account for the existence of freshwater ecosystems that have stable conditions across the year. Other freshwater ecosystems that present harsh seasonal variations, with lakes that completely dry in the summer or that freeze completely in the winter, implies major differences on the biotic communities of different types of lakes. Differences in the chemistry are also noticeable. In mainland aquatic environments there is great variation of pH with the existence of alkaline lakes with pH over 11 to acidic peat bogs with pH below 3.0. This different abiotic characteristics made possible the evolution of organisms that are able to survive in diluted waters with a wide spectrum of ions proportions in the water, being that osmoregulatory adaptations are a major importance to the maintenance of aquatic food webs in these freshwater ecosystems (Cole 1976).

Different lakes with different dimensions are very distinct. There are differences in water renewal time between different lakes mainly due to the ratio between lakes' volume and its catchment area, their physical properties or even due to regional climate, evapotranspiration and rainfall rates. (Schindler 1991). High water renewal rates associated with geological settings with low rates of ions exchange tend to promote very low concentration of solutes in lakes (Schindler 1991). Chemical differences between these waterbodies may modulate biota communities. In fact, regulation of trophic webs in lakes are associated with solutes in the water and can be explained accordingly to *bottom-up* and *top-down* theories (Jeppesen et al. 1997; McQueen & Post 1986). *Bottom-up* theory states that biomass in an ecosystem is controlled from the base of the food web, by the producers, and *top-down* theory states that biomass is controlled by the consumers at the top of the food web. However, in freshwater ecosystems it appears that the theories complement each other in a manner that *bottom-up* regulation seems to be stronger at low trophic levels, weakening as trophic level increases, while *top-down* regulation gain weight at the top of the food web (McQueen & Post 1986). So, high nutrient levels in lakes may lead to an overall increase in the primary producer's biomass, emphasizing the role of *bottom-up* regulation in the food web (Du et al. 2015; McQueen & Post 1986). However, it is worth noticing that top-down control seems to be stronger in shallow lakes (Jeppesen et al. 1997). This dynamic relation may be important to understand communities' dynamics and their food webs in lakes.

The depth of a lake is also an important factor differentiating the aquatic communities. In deep lakes, light is not able to reach the bottom restraining algal growth to the superficial layers, being that organisms in this lake zone are mostly dependent on the organic matter precipitation from the lake surface. On the other hand, in shallow lakes the light is able to spread and reach all lake zones promoting algal and plant growth, enabling shallow lakes to support a greater amount of consumer organisms' biomass (Mann 1991). In fact, even in deep lakes, most vital activity occurs in shallow water zones with low depth where light reaches and nutrients are photosynthetically fixated, thus augmenting organic matter present and promoting the formation of more richness communities (Schindler 1991).

Natural Ponds

Throughout the planet, in all biomes, several small waterbodies are formed across the landscape and are yet not well-known. These small waterbodies, addressed as ponds, are generally small and shallow natural waterbodies (1 m^2 to about 5 ha) capable of retaining water temporarily or permanently (Céréghino et al. 2008; De Meester et al. 2005). It is not easy to differentiate ponds from small lakes because there are many resemblances in terms of structure and function. On the other hand there is a slow gradual transition from pond to lake ecosystems, being that each case may be evaluated differently and accordingly to other factors like the structure of the communities, the presence or absence of benthivorous fish and the impact of the wind in the system (De

Meester et al. 2005). However, generally ponds have higher rates of matter and organisms exchange with nearby terrestrial environment even though they are usually more isolated and usually have a lack of fish populations mainly due to their size and possibility of freezing during winter or drying during summer. Besides that, shallow ponds have low input and output of water, what makes the water relatively stagnant, giving importance to the impact of sediments in the water nutrient content (Söndergaard et al. 2005).

Despite not being well studied, ponds have gradually gained scientific importance in the last few years, and are now seen as a major interest in a global climate changing scenario. Natural ponds present many ecosystems services that can represent sustainable solutions regarding some climate change problems, solutions like the mitigation of diffuse pollutants and carbon sequestration. Besides that, ponds seem to be good biodiversity hotspots harboring communities highly diversified in species and in functional characteristics (Céréghino et al. 2014; De Meester et al. 2005).

Ponds structure and function are yet poorly understood. De Meester et al. (2005) proposed that ponds can be seen as attractive model systems for hypothesis-testing in fields like ecology, nature conservation, and evolutionary biology. They occur in a wide variety of pond types and are abundant throughout the globe, what allows studies along any ecological gradient. The great contact zone between the aquatic and terrestrial environment allows ponds to suffer impacts from anthropogenic pressures, what makes them ideal tools to track changes in the overall ecosystem health. Ponds are considered aquatic suitable patches in an unsuitable matrix, making them also good model systems for research on metapopulations and metacommunities. Their small size and simple structure allow the application of repeatable and representative sampling methods, which allows hypothesis testing *in situ* or with whole-ecosystem approaches. Lastly, pond ecosystems can be simulated in mesocosmos experiments, enabling large-scale replication and the test of anthropogenic stressors in the laboratory.

In addition to the high scientific value of this specific ecosystems, differences in the catchment areas of natural ponds are responsible for the great variations noticed in the water chemistry. A single pond can have great physical and chemical variations mostly related to the composition and texture of the sediments, the activity of the vegetation, the sedimentation, and processes of matter decomposition (Bazzanti et al. 2010). This heterogeneity of environmental conditions appears to be the main factor connected to the communities compositions when in comparison with regional or biological drivers, thus promoting the formation of different mesohabitats in each single pond (Davies et al. 2008; Hill et al. 2017) what makes these waterbodies suitable to harbor great and unique biodiversity, being of major conservation importance. It has been demonstrated that ponds contribute greatly to regional biodiversity. They usually present high species richness, having considerably more unique and rare species that present greater diversity of ecological strategies and biological traits than other types of waterbodies like rivers (Céréghino et al. 2012; Davies et al. 2008; Williams et al. 2004). As theorized by Scheffer et al. (2006), species richness in small waterbodies is mainly influenced by their isolation and small size. These two factors inhibit potential colonization by fish (that negatively influences the invertebrate community due to competition and predation pressure), which contributes not only to a lesser pressure in macroinvertebrates populations, but also to a better vegetation development. Therefore, higher macrophyte abundance, providing food and habitat structure benefic to amphibians, macroinvertebrates and water birds diversity in these freshwater ecosystems. On the other hand, isolation could reduce local diversity of water invertebrates' due to dispersal difficulties. However, some species of macroinvertebrates possess good dispersion abilities (Bilton et al. 2001) being able to overcome this setback and thus enabling communities' differentiation. This is responsible for high regional diversity of these systems when in comparison to other aquatic systems, even though close ponds may be similar (Scheffer et al. 2006). A lot of species can potentially colonize these small waterbodies, but there are some that were only found in them, these are unique and many times endemic species of amphibians, dragonflies, and aquatic plants, what reinforces the conservation value attributed to these natural ponds (Oertli et al. 2005).

Alpine Ponds

Ponds have a wide geographical distribution, appearing at different altitudes also. As we explore mountain ecosystems, it is noticeable that there are environmental differences while comparing to other ecosystems. As altitude increases, UV radiations intensifies, daily air temperature decreases, annual precipitation increases and the growth season period diminishes (Hinden et al. 2005; Körner 2008) are same specific characteristics of this alpine ecosystems. At high altitudes, an alpine zone emerges with unique characteristics in a singular ecosystem. The extreme seasonality with very cold and windy winters with snowfall and ice formation and with short-termed summers associated with a limited nitrogen and phosphorous availability have a great impact on the annual biomass production that occurs mostly in the two months of the favourable season (Elser et al. 2009; Körner 2007).

In high mountain ecosystems, it is common the formation of lakes and ponds in the alpine zone. Due to the inaccessibility and the harsh environment in this zone, these small waterbodies are usually isolated, with very few and punctual anthropogenic disturbances, being mainly found in pristine conditions and in an oligotrophic state (Boavida & Gliwicz 1996; Hinden et al. 2005; Körner 2008). Alpine ponds have unique characteristics mainly due to their extreme environmental conditions but also due to their formation type. These ponds are mainly formed by the accumulation of water from precipitation, from the meltdown of the snow and ice cover, from the water flux of subterranean water systems or even from alpine streams, in more rare cases (Bengtsson 2012a; Dokulil 2005). Although dependable of the catchment area properties, some general characteristics of alpine ponds might include low water mineralization, low temperatures, low nutrient concentration and usually have neutral or slightly acidic pH (Bengtsson 2012a). Seasonality is of major importance in small waterbodies in alpine zones. Overall, they are subject to low temperatures but with a great variance throughout the day. These typically cold temperatures promote snowfall and ice forming at the waterbody surface during most part of the year. This conditions alternate with shorten periods of the growing season, being a limiting factor for biodiversity fixation (Hinden et al. 2005; Körner 2008).

The adverse and extreme conditions described in alpine ponds are obstacles to the fixation of biodiversity and have a huge impact on the local biota (Régis Céréghino et al. 2012). Nonetheless, in a net of several alpine ponds, each one with its own characteristics, can provide high heterogeneity of environments increasing the potential in biodiversity of theses ecosystems (Hamerlík et al. 2014), since that species richness patterns are associated with habitat characteristics as water chemistry, vegetation cover, presence or absence of predators and connectivity between ponds (Scott A. Wissinger et al. 2016). All factors mentioned above provide the colonization of a few number of well-adapted species that possess unique anatomic and physiological features that allow their survival and proliferation in these extreme conditions (Bale 1996; Lencioni 2004). Adaptations to the low temperatures, to high UV radiation, to the strong winds and to the scarce of nutrients are needed to subsistence in these conditions. An alteration in communities composition across an altitudinal gradient is noticeable, with a decrease of eurythermal taxa and a complementary increase in cold-adapted stenotherms (Rosset & Oertli 2011). Some cases of zooplanktonic species possessing adaptations to the high radiation as pigmentation, DNA repair mechanisms or even behavioral methods as avoidance have already been documented (Scott A. Wissinger et al. 2016). Besides that constrains, biota of alpine ponds needs to be prepared to survive during the long unfavorable period, adopting life strategies that allow their maintenance and synchronizing their life cycles to take the most advantage of the favorable season (Lencioni 2004). In some species of limnephilid caddisflies it was observed an adult diapause that allows a delay in oviposition until late autumn in alpine aestival ponds (S. A. Wissinger et al. 2003).These different features of alpine ponds make pressure in the biota. This pressure enables the emergence of new and unique communities adapted to live in the extreme conditions of the alpine ponds. Thus, these waterbodies may be sanctuaries of unique and rare biodiversity, sometimes harboring endemic species (Čiamporová-Zaťovičová et al. 2010; Clements et al. 2016).

The small size and simple biotic structure of alpine ponds make them ideal sites for conduct ecological studies and for monitoring alterations due to climate change through time (De Meester et al. 2005; Oertli et al. 2008). Alpine ponds are very sensitive to climatic changes, being greatly impacted by anthropogenic pressures like tourism, acidic deposition, and poor agroforest practices. Vulnerability to acidification in alpine ponds with non-carbonate bedrock in the catchment area was already described (Curtis et al. 2005; Skjelkvåle & Wright 1998). Acidification processes may also reduce dissolved organic carbon content in waterbodies, what allows an increase in the penetration of UVB rays (David W. Schindler et al. 1996). This factors will affect biota like microcrustaceans, malacostracans, molluscans, mayflies and caddisflies, with consequent cascade effects in the food web (Bradford et al. 1998). Murphy et al. (2010) demonstrated that alpine ponds of the Canadian Rockies are even more nitrogen limited than alpine lakes and thus more sensitive to anthropogenic nitrogen deposition, what allows an early detention of irregular nitrogen deposition. All this factors can be used as a tool to alert in early stages to alterations that are occurring on the long run (Toro et al. 2006; Watson & Haeberli 2004).

As the overall temperature of a region rises, alpine ponds and lakes catchment areas will experience defrost sooner in the spring. With the shortening of the snow-cover period, pond will be less time covered in ice and catchment sites at high altitudes will have lesser snow-cover as the overall temperature increases (Skjelkvåle & Wright 1998; Thompson et al. 2005). Global warming can also promote changes in high altitude ecosystems. These ecosystems turn more suitable to harbor new species usually seen in ponds at lower altitudes thus leading to an increase local and regional species richness but with the loss of stenothermal species (R. Céréghino et al. 2008; Oertli et al. 2008). The changes in species geographical distributions and in presence/absence patterns are good warning signs against changes in the overall ecosystem.

Furthermore, alpine ponds have a great variation in harshness and drying regimes, what makes them ideal environments for different research studies. The interaction between abiotic and biotic parameters in community structure; the role of disturbances and ecological interactions in local species diversity and richness; and the importance of dispersion in metacommunities dynamics at different altitudes are some examples (Scott A. Wissinger et al. 2016).

Freshwater invertebrates

Recently the scientific interest in natural ponds is increasing (Régis Céréghino et al. 2014; M J Hill et al. 2016; Matthew J. Hill et al. 2017; Serrano et al. 2017; Strachan et al. 2014). From the wide array of organisms used in biomonitoring, benthic macroinvertebrates gained popularity due to many advantages when was used. They are ubiquitous (suffering from perturbations in different habitats), usually have great species richness, what implies different responses in face of perturbations. Many of them have low dispersal ability, what enables a confident delimitation of the study area and their life spawn allows the detention and monitoring of temporal changes (Mandaville 2002).

Aquatic benthic macroinvertebrates are important and sensitive organisms to study in ecology works. They show different dispersal, synchronization, reproduction strategies and can adapt themselves to the environmental conditions with changes in their behavior, morphology and physiology (Lencioni 2004; Verberk et al. 2008). For example, there are aquatic invertebrate species that are found in environments with harsh conditions, tolerating scarce food availability by being able to assimilate food rather efficiently and tolerating high acidity or alkalinity values by expending a great amount of energy to maintain the homeostasis (Verberk et al. 2008). The different life-history strategies of these organisms have diverse functional implications and enable them to colonize a wide array of environments. They represent different solutions to specific ecological problems, allowing macroinvertebrates to be found in different ecosystems allowing the possibility of colonization of a waterbody by distinct orders of aquatic invertebrates (Verberk et al. 2008).

As previously mentioned, natural ponds present a great variety of habitats that are colonized by different sets of aquatic invertebrates. Several factors such as the abundance of organic matter, a wide array of food sources, great habitat stability, availability of refuges against predation and overall exceedingly favorable environmental conditions verified in ponds are the main reason of high macroinvertebrates specific richness in these aquatic systems (Bazzanti et al. 2010). This heterogeneity and the consequent array of benefits promote colonization by different families of macroinvertebrates, what composes a highly diversified macroinvertebrate community as shown by Céréghino et al. (2012). The authors showed that invertebrates of ponds have a greater diversity of ecological strategies and biological traits in comparison with macroinvertebrates from lakes, rivers or streams. Species of Trichoptera, Plecoptera and Ephemeroptera are specialized to survive in cold and oxygen-saturated waters like *Nemurella picteti* and *Hesperophylax occidentalis* that are typical from lotic systems were documented in alpine ponds in the Swiss Alps and in the Colorado Rockies respectively (Scott A. Wissinger et al. 2016). There is also a change in voltinism in high altitude invertebrates in comparison with their low land counterparts (Scott A. Wissinger et al. 2016). Species like *Callicorixa audeni* and *Cenocorixa bifida* can only complete one generation per year in alpine ponds while it is normal two or more in low land waterbodies; and the dragonfly *Stomatochlora semicircularis* which larvae takes only two years to develop in low altitude ponds while taking up to four years in alpine ponds (Scott A. Wissinger et al. 2016).

However, macroinvertebrate distribution in ponds is not regular by all habitats. Central habitats without vegetation and characterized by fine grain sediments, high nutrient values and low oxygen content seem to have lower abundance and faunistic diversification. On the other hand, other substrates like zones dominated by macrophytes that are capable of providing good conditions like sediment stability, refuge against predators, good oxygenation, food abundance due to plant senescence and algal development seems to have an higher plankton abundance (Bazzanti et al. 2010).

In temporary ponds, many invertebrates show traits associated with species of "r" strategy, with quick growth, small size, short lifetime, high dispersion power and generalistic feeding, all associated with a lack of competitive skills (D. D. Williams 1997). Migration may also occur as a response to the seasonal hard conditions. Adults may disperse in search for new ponds formed on spring after withstanding winter in a perennial waterbody, so they can lay eggs in a safer environment for the larvae to grow (D. D. Williams 1997). Active and rapid dispersal of insects is a way of finding new suitable environments in the unfavorable period, but passive migration may also occur in small species that can travel with the wind or synchronize their life cycle for using a migration vector (Sim et al. 2013; D. D. Williams 1997) Other species are specialized to produce resistance eggs like the limnephilid caddisflies that produce semiterrestrial eggs and the lestid damselflies that produce endophytic eggs capable of being dormant all winter season (Scott A. Wissinger et al. 2016). Behavioral adaptations are also noticed in aquatic macroinvertebrates. To survive in temporary ponds, these invertebrates are mostly generalistic/opportunistic to surpass the scarce of food availability (D. D. Williams 1997). Occupation of different micro refuges by invertebrates was also reported by Sim et al. (2013) in order to thrive through draught periods in temporary wetlands. Reproduction changes are also common, being parthenogenesis an alternative already found in different species of Diptera, Ephemeroptera and Plecoptera, namely in northern regions (Lencioni 2004). Other characteristics were found in aquatic vertebrates to withstand harsh conditions like differences in dispersal methods, times of development, reproduction efforts and tolerance trade-offs (Lencioni 2004; Verberk et al. 2008).

In comparison with low land biota, invertebrates of alpine ecosystems have morphological adaptations as melanism to protect against UV radiation and to facilitate heat accumulation. Other alterations may be occurring is the decrease of body size. This situation not only allows faster growth and development (useful due to the small favorable season), but also reduces the quantity of food needed, what is fundamental in these types of scarce environments and increases the opportunity of finding suitable microhabitats. Furthermore, the reduction of flight apparatuses with the reduction of wing size (brachyptery) or total loss of the wings (aptery), reducing the contact area exposed to low air temperature and to the strong winds of alpine and polar regions improve the success of this species in these environments (Lencioni 2004). Other physiological mechanisms were already described such as quiescence and diapause, that are mechanisms that allow these aquatic invertebrates to synchronize their development to the season variations occurring in the ecosystem (Lencioni 2004; Verberk et al. 2008). In quiescence process animals reduce their activity when in harsh conditions, as low temperature or lack of food (Verberk et al. 2008). In a diapause mechanism, the organisms state a rigid physiological modifications to pause or delay the development to bypass long adverse periods that are predictable or recurrent (Tauber & Tauber 1981; Verberk et al. 2008). Aquatic invertebrates are also able to survive under long extreme cold periods with physiological and biochemical adaptations to tolerate freezing pressures or to avoid them. These organisms can hibernate so it becomes possible to tolerate extremely low temperatures by freezing all extracellular fluids or even whole tissues in the most extreme cases. Another mechanisms to avoid freezing is conduct by entering in a supercooling state, maintaining their fluids in a non-freezing state by synthesizing cryoprotectants and anti-freezer molecules from lipids and glycogen in the hemolymph (Bale 1996; Lencioni 2004).

Aims

There is a lack of scientific information for the alpine areas in Portugal, namely what referring to the alpine ponds. Thus, the necessity to gather data about these waterbodies arises. To bridge this gap, the main scope of this work is to study the dynamics of the macroinvertebrate communities of five alpine ponds in Serra da Estrela. Additionally, the obtained information was used to perform a pond classification accordingly to their ecological quality. A second objective of this study was to understand which abiotic parameters modulate macroinvertebrates occurrences and understand seasonal dynamic variations of their communities in these alpine ponds through the year.

Materials and methods

Study area

Serra da Estrela (N 40º 20’, W 7º 25’) is located in northern-central of Portugal and is the highest mountain massif in Portugal mainland, reaching 1993 meters high. As part of the Iberian Central Zone, this mountain massif elongates in an NNE-SSW direction, being about 45 km long and 20 km wide (Marques et al. 2006; Migoń & Vieira 2014; van der Knaap & van Leeuwen 1995). Serra da Estrela characterized by a Mediterranean climate, what is noticed by the hot dry periods of the few summer time and with a wet season with rainfall and snowfall (2500 mm a year in the peak, 2000 mm a year in the plateaus) between October and May (Daveau et al. 1997; Vieira et al. 2009). The ecological and geological importance of this area, with high mountain and alpine ecosystems, is recognized with the delimitation of the “Parque Natural da Serra da Estrela (PNSE)” by the Decree-law 557/76 of 16 July 1776. Serra da Estrela geology is deeply marked by granitic rocks in the center of the massif and by metasedimentary complexes in the periphery area. Changing environmental conditions and the harsh associated processes molded granite geomorphology of this region (Ferreira & Vieira 1999; Migoń & Vieira 2014).

For this study five natural alpine ponds of Serra da Estrela were selected (Table 2) based on the differences between their catchment proprieties, habitat types, hydrological regimes, and geological differences (See Appendix III). All ponds are considered alpine once they are located above 1700 meters. The physical characteristics revealed that the ponds have a total area ranging between 0.5 to 1 km^2 and a depth comprehended from shallow waters (0.5 m) up to 3 meters. Ponds 3, 4 and 12 are located on the north side of the only road in the area whereas ponds 7 and 10 are located in south side of the road (Figure 1 and Table 2). This road and the use of salt for deicing in winter may have an impact in water quality of nearby waterbodies as already shown by Rodrigues et al. (2010) . The ponds studied may also suffer a similar impact since there is water run off to south of the road (to ponds 7 and 10). This possible impact should not be notice in ponds north of the road (P3, P4 and P12).

Figure 1 - Study ponds location (3, 4, 7, 10, 12) in Serra da Estrela Natural Park.

Table 2 - Study ponds coordinates (first in geodesic format and second in decimal geodesic format).

Pond code	Latitude	Longitude	Altitude (m)
3	40° 20′ 16.962″ N	7° 36′ 41.173″ W	1834
	40.338045°	-7.611437°	
4	40° 20′ 17.081″ N	7° 36′ 27.482″ W	1822
	40.338078°	-7.607634°	
7	40° 20′ 4.542″ N	7° 37′ 25.662″ W	1749
	40.334595°	-7.623795°	
10	40° 20′ 8.855″ N	7° 37′ 25.72″ W	1736
	40.335793°	-7.623811°	
12	40° 20′ 21.077″ N	7° 36′ 54.22″ W	1848
	40.339188°	-7.615061°	

Three sampling periods are conducted between 2016 and 2017, whereas different abiotic and climatic conditions. The first sampling campaign was conducted in summer of 2016 after the ponds defrost. The second sampling period was accomplished in autumn 2016, before the arrival of the cold and the first snows, and the last campaign was conducted in the spring of 2017 after the pond defrost.

Sampling methods

In situ procedures

In each pond, a several of *in situ* parameters were measured using a multi-parameter probe (Multi 350i): temperature (ºC), dissolved oxygen (mg/L and %), pH, conductivity (µS/cm), and Total Dissolved Solids (mg/L). Additionally, water samples were collected using 1.5 L of plastic bottles for further analyses in the laboratory (photosynthetic pigments, suspended solid particles, turbidity, dissolved organic carbon and concentration of phosphates, nitrites, nitrates, ammonia).

Macroinvertebrates sampling was conducted according to standard protocols described in Water Framework Directive (transposed into Portuguese guidelines in http://www.apambiente.pt/dqa/invertebrados-bentonicos.html). According to this it was performed a composed sampling procedure in which there were performed three drag repetition using a net with 0.5 mm mesh and 25 cm of wide (Figure 2). The composed sample was dispersed in each pond considering that all the habitats present were represented, accounting the heterogeneity of all ponds as suggested by INAG (2008). The macroinvertebrates samples were preserved using formaldehyde (40%) until further analyses.

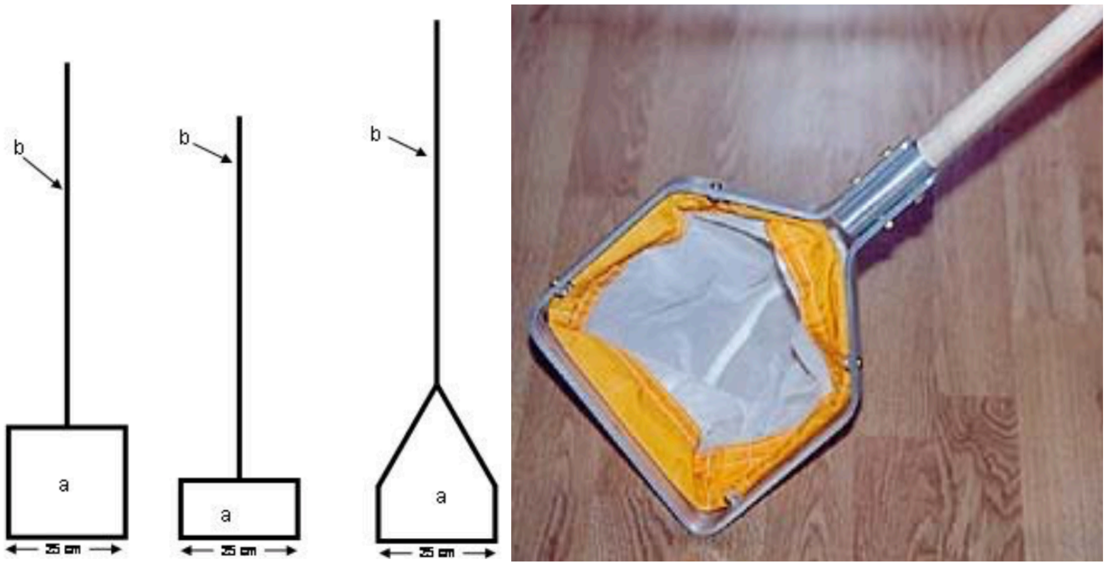

Figure 2 - Scheme of the macroinvertebrate sampling net and a photo of the same (Adapted from INAG (2008)).

Laboratorial procedures

In the laboratory water samples were posteriorly treated to achieve several physical and chemical parameters for each pond. The water sample was divided into aliquots according to parameters determination. One aliquot of each water sample was filtered while still fresh (up to 8 hours after sampling), for determination of chlorophyll *a* and total suspended solids. For this determination, filtration cups were used and the water samples were forced to pass through a fiberglass filter (47 mm diameter; 1,2 µm pore) using the pressure generated by a suction pump (Strickland & Parsons 1972). The filter retains all the *seston* of the sample (e.g. particulate matter and plankton namely phytoplankton), which was used to quantify the total of suspended solids and determine the chlorophyll *a* content (Chl *a*). The water resulting from filtration process was used to determine the dissolved organic carbon (DOC). The remaining unfiltered samples were used to assess other laboratory parameters: turbidity, ammonia, nitrates, nitrites and phosphorus concentration. In the laboratory, the determination procedures were conducted according to previous guidelines that are described above:

- **Chlorophyll *a***

Chlorophyll *a* (Chl *a*) is a photosynthetic pigment present in photoautotrophic organisms like microalgae and cyanobacteria, and its quantification may be used as an approximation of the algal biomass and of the aquatic systems primary production (Lorenzen 1967).

To determine the chlorophyll *a* content in each sampling site, a filter used in the filtration process were put in a falcon tube with 5 mL of alkalized acetone (90%). The tubes were covered with aluminum foil for the process could occur in total darkness in order to avoid chlorophyll photo-oxidation. The extraction process was conducted at 4 ºC approximately overnight (15 to 20 hours; Chl *a* extraction was too low thenceforth). After this period, the tubes were centrifuged at 3500 RPM for 5 minutes. After the centrifugation, the supernatant was collected and read the absorbance in a spectrophotometer at 750 nm and 665 nm. After these readings samples were acidified with 2 drops of diluted hydrochloric acid (HCl 0.1M) and measured again at the same wave-length, 3 to 4 minutes later. The acidification with HCl promoted the degradation of the chlorophyll without affecting the phaeopigments that resulted from the chemical reaction. Chl *a* content was assessed as follows equation:

$$Chl\ a\ (\mu g L^{-1}) = \frac{26.7 \times (E665_0 - E665_a) \times v}{V \times l}$$

In which $E665_0$ is the difference between the absorbance at 665 nm and 750 nm, $E665_a$ is the difference between the absorbance at 665 nm and 750 nm after the acidification, v is the volume of acetone in millilitres used for the extraction, V is the filtrated water volume and l is cuvette's optical path.

- **Dissolved Organic Carbon**

Dissolved organic carbon (DOC) is an important parameter to assess once the organic carbon dissolved in the water attenuates solar radiation in freshwater habitats and interacts with inorganic components, changing their availability (Williamson et al. 1999).

This parameter can be assessed using an indirect method based on spectrophotometric absorption. The filtrated water samples were read in a spectrophotometric at 320 nm of absorbance. The value measured was used to calculate the absorbance coefficient, which is used to stipulate the colored fraction of the dissolved organic carbon according to the following equation was used:

$$\varepsilon_{320} = \frac{2.30 \times ABS_{320}}{l}$$

In which $\varepsilon_{320}$is the absorption coefficient (m^{-1}), ABS_{320} is the absorbance at 320 nm and l is cuvette's optical path in meters.

- **Turbidity**

A high concentration of suspended solids and chemical substances in the water decrease the light transmission in a water column since it promotes light absorption and/or dispersion, which affects the primary producers (Brower et al. 1998).

In order to obtain information about sample's turbidity, an indirect method was used to assess the absorption coefficient (proportional to turbidity) based on spectrophotometric reading. Absorbance was read at 450 nm for each water sample and the absorption coefficient was calculated according to the equation:

$$\varepsilon_{450} = \frac{2.30 \times ABS_{450}}{l}$$

In which ε_{450} is the absorption coefficient for each sample (m^{-1}), ABS_{450} represents the absorbance at 450 nm and l is the cuvette's optical path in meters.

- **Nitrates, nitrites and ammonia concentration**

In freshwater ecosystems, nitrates, nitrites, and ammonia are the most important nitrogen states and can be biochemical converted into one another (APHA 2005). In lake water, nitrogen normally varied from 0.01 to 1.0 mgL^{-1} (Davis & Simmons 1979).

Regarding these nitrogen states, nitrites are in an intermediate state in the oxidation of ammonia and the reduction of nitrate (APHA 2005). Ammonia appears naturally in water systems, while nitrates are usually scarce.

To access the amounts of the parameters described above, a photometric test was performed using Spectroquant Multi Colimeter. Procedures from tests 1.14773, 1.14752 and 1.14776 were used to quantify nitrates, ammonia and nitrites, accordingly to standard procedures.

Macroinvertebrates community

In the laboratory, the macroinvertebrates samples firstly were screened in order to separate macroinvertebrates from the debris. After this sorting, the organisms were identified using a magnifying glass and recurring to identification keys (Tachet 2000). The identification was made to the lowest taxonomic level whenever as possible in order to better achieve the diversity and richness of each pond.

Statistical analysis

A Principal Component Analysis (PCA) were conducted to achieve relations between physical and chemical parameters in each pond along the sampling period. This multivariate analysis allows to access the combinations of variables that explain the larger amount of variation in the dataset (Fowler et al. 1998).

Characterization of macroinvertebrates communities was made using several indexes. Abundance was determined by counting the total number of individuals in each sample. The taxonomic richness was obtained by checking the number of different taxa in each sample. Shannon-Weaver index was also calculated in order to measure the diversity of a sample, using the equation:

$$H' = \sum_{i=1}^{S} pi \ln{(pi)}\,, pi = \frac{n_i}{N}$$

where H' is Shannon-Weaver index value, n_i the number of individuals of "I" species and N the total number of individuals in the sample.

Pielou's evenness shows the equitability of the community in the sample, allowing to see if there are dominant species or if they're equality distributed in the community. Evenness values vary between 0 and 1, and higher values are associated with more even communities that doesn't show a dominant species. Pielou's evenness index was calculated according to the equation:

$$J = \frac{H'}{H_{máx}}, H_{máx} = \ln{(S)}$$

where J is Pielou's evenness index value, H' is Shannon-Weaver index value and S is richness.

One-way ANOVAs were computed to access variations in the indexes calculated between pond communities throughout the seasons. When one-way ANOVA showed significant variation in the data, t-tests were conducted to test which sample period was different.

To check for relations between physical and chemical water parameters and communities' variations, a correlation test was performed. As the dataset for each parameter and for the indexes calculated were not normally distributed, non-parametric Spearman's correlation tests were performed.

A Correspondence Analysis (CA) was performed to check for aquatic invertebrates' distribution patterns across the sampling ponds over the sampling periods.

IBMWP (Iberian Biomonitoring Working Party) and IASPT (Average Score Per Taxon) indexes were also calculated. These indexes use different macroinvertebrate families as bioindicators, counting the families present in a sample and attributing a "score" value from 1 to 10 to each family accordingly to their sensibility, when higher values present the more sensible families (see Appendix II). Although IBMWP and IASPT indexes are used to access rivers water quality in Iberian rivers, their use can provide some useful information about communities' dynamics. IBMWP is based on BMWP (Biological Monitoring Working Party) and is a rapid method that uses the different degrees of tolerance of different macroinvertebrates families to pollutants to access water quality in the Iberian Peninsula (Alba-Tercedor & Sánchez-Ortega 1986; Hellawell et al. 1978). IASPT is also an Iberian index and can be used when IBMWP values are similar because it can provide information about the score of the *taxa* present in each sample, independently from local family richness (Alba-Tercedor & Sánchez-Ortega 1986; Armitage et al. 1983). Higher values of IASPT are associated with sites containing high scoring *taxa*.

In Portugal, in river ecosystems, EQR (Ecological Quality Ratio) can be calculated using benthic macroinvertebrates data. In the north of Portugal, EQR can be calculated as the reason between $IPtI_N$ (Índice Português de Invertebrados Norte – Northern Invertebrates Portuguese Index) value and the median of the reference values obtained for that index in reference sites of a determined typology. Although this index is used typically for rivers, its use can provide meaningful insight about the ecological quality of the waterbodies studied. As the studied ponds are situated in the north at high altitudes, the typology of the river that was best adapted to the local study site was M type (Mountain rivers of the north). EQR results are used to qualify a waterbody based on a qualitative scale (Bad, Poor, Fair, Good or Excellent) (for EQR and $IPtI_N$ reference values and $IPtI_N$ formula, see INAG 2009, Appendix A).

Results

Water Chemistry

Table 3 presents the values of physical and chemical parameters measured in each pond over the sampling period (June, October and April). It is noticeable in all the sampling ponds the water temperature seasonality. In the summer period (June), the temperature values are the highest (20 to 25 °C) for all the sampling ponds, and in October, the temperatures measured are the lowest (all between 10 and 11 ºC). In April, after the snow break and in the beginning of the growing season s, an increase in water temperatures in all ponds was observed. All the ponds show neutral to slightly acidic pH values. Moreover, all ponds except P4 seem to have slightly lower pH values in October. Both dissolved oxygen (mg/L and %) seem to have high values in all ponds, with an increase of values recorded in April. Conductivity and TDS values have a normal distribution and are highly correlated (Pearson's correlation = 0.999, $p < 0.05$) and both present low values in all the samples. However, pond 10 had always the highest conductivity and TDS values in comparison to the other ponds.

Table 3 - Results of physical and chemical parameters measured *in situ*.

Ponds	Months	Temperature (ºC)	pH	Conductivity (µs/cm)	TDS (mg/L)	O2 (mg/L)	O2 (%)
P3	**June**	25.2	7.50	8.50	9.00	9.40	115
	October	10.0	4.25	16.5	16.0	5.59	51.5
	April	19.1	6.14	8.80	9.00	9.90	133
P4	**June**	20.0	7.67	4.30	4.00	8.20	91.5
	October	10.0	7.58	6.00	6.00	7.63	69.4
	April	15.7	6.80	5.80	6.00	8.39	105
P7	**June**	20.9	5.53	9.30	9.00	7.00	82.0
	October	11.0	4.33	11.7	12.0	6.52	61.9
	April	15.8	5.40	6.60	7.00	8.42	105
P10	**June**	20.4	6.82	105	101	9.80	102
	October	10.5	4.80	106	108	7.99	74.0
	April	14.3	6.22	82.4	84.0	10.4	124
P12	**June**	20.8	7.47	6.30	6.00	6.95	82.0
	October	11.0	4.46	10.7	11.0	7.23	68.8
	April	13.9	6.02	6.20	6.00	8.38	101

Laboratory water analysis is shown in table 4. Turbidity values observed are generally low throughout the sampling period. Coloured dissolved organic carbon (CDOC) follows the same trend, with higher values in ponds P3, P10, and P12 in April. The nutrients values quantified are low (some even below the calorimeter detection level appearing as BDL in table 4) for all the ponds. Chlorophyll *a* content was also variable with a maximum recorded in pond 7 in October. However, all ponds have low content in chlorophyll *a*, being all values typical from oligotrophic waters.

Table 4 - Results of water laboratory quantifications. ND – Non-Detected, BDL – Below Detected Limit (Nitrates < 2.2 mg/L, nitrites < 16 µg/L and ammonia < 0.03 mg/L).

Ponds	Months	CDOC (m^{-1})	Turbidity (m^{-1})	Nitrates (mg/L)	Nitrites (µg/L)	Ammonia (mg/L)	Chlorophyll a (µg/L)
P3	June	0.092	0.0276	BDL	19	BDL	1.187
	October	0.156	0.0414	5.9	BDL	0.03	0.534
	April	0.478	0.3933	BDL	92	BDL	3.204
P4	June	0.005	ND	7	20	0.03	ND
	October	0.021	0.069	6	BDL	0.07	0.534
	April	ND	ND	BDL	31	BDL	ND
P7	June	0.078	0.0253	7.3	27	BDL	1.820
	October	0.159	0.0575	6.5	25	0.09	5.547
	April	0.009	0.0023	BDL	71	0.07	1.335
P10	June	0.018	0.0069	BDL	26	0.07	0.801
	October	0.046	0.0046	7.7	26	0.05	0.267
	April	0.430	0.0046	BDL	49	BDL	4.272
P12	June	0.041	0.0230	BDL	19	BDL	0.600
	October	0.083	0.0069	3.4	37	0.03	0.534
	April	0.386	ND	BDL	59	BDL	ND

To assess relations between the physical and chemical parameters in each pond over the sampling period a Principal Component Analysis (PCA) was computed (Figure 3). Component, or axis 1 explains 33.27% (eigenvalue=3.99) of the variation in the data and component 2 explains an additional 20.42% (eigenvalue = 2.45). PCA, makes a separation of three distinct groups. The group 1 has all the samples collected in October (solid circle). The group 2 has four of the June samples except pond 10 (dotted circle) due to the high overall TDS and conductivity values found in this pond. The group 3 has almost all April samples except pound 4 (dashed circle) probably due to the low values of CDOC, Turbidity, Nitrates, ammonia and chlorophyll *a*, most with non-detected or below colorimeter detention level values (Figure 3). These groups were separated according to waterbody properties that were greatly influenced by seasonality, with higher ammonia and nitrates concentrations in October samples. June present the highest values of temperature and pH. While in April, the ponds are influenced by high values of nutrient, namely phosphates and nitrites, and values of dissolved oxygen.

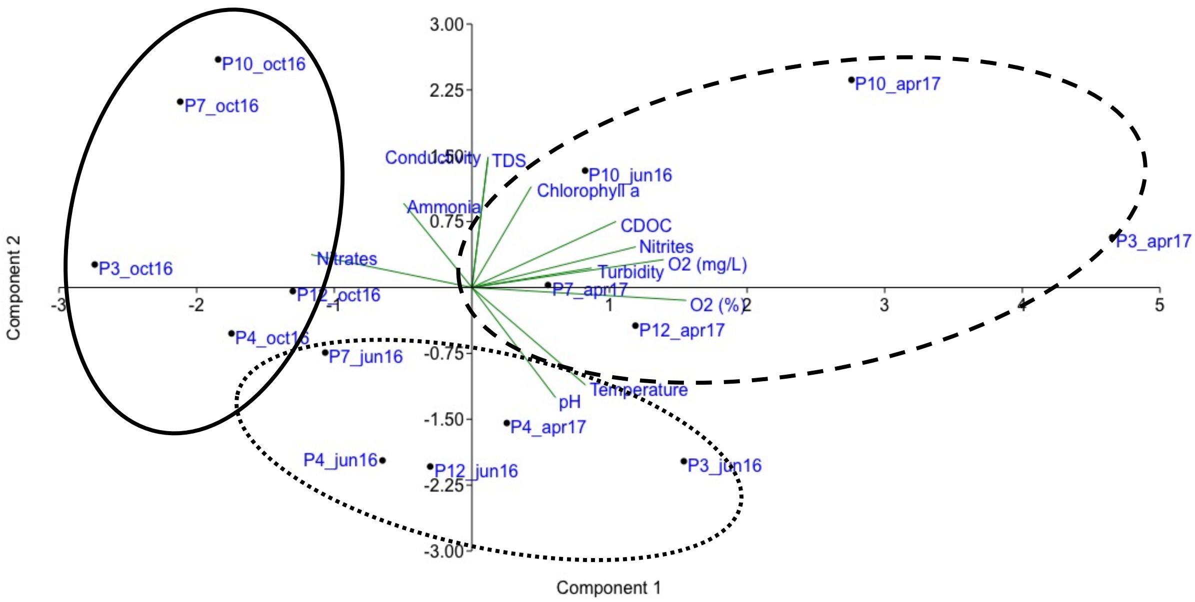

Figure 3 - Principal Component Analysis (PCA) to assess relations between physical and chemical parameters in each pond over the sampling period.

Macroinvertebrates Communities

All macroinvertebrates present in each sample were counted and identified to the lowest taxonomic level possible (see Appendix I). Values of macroinvertebrates abundance for each pond and sampling period are presented in Figure 4. No significant differences along the seasonal gradient (p = 0.278) were recorded for abundance values. Nonetheless, pond 10 had high abundance variation in April. Regarding pond 3, a seasonal variation was also observed with a highest abundance value registered in April too. In pond 7 a decrease of abundance was observed over the sampling period with the higher level recorded in June. Pond 12 seems to have the more stable abundance across all seasons.

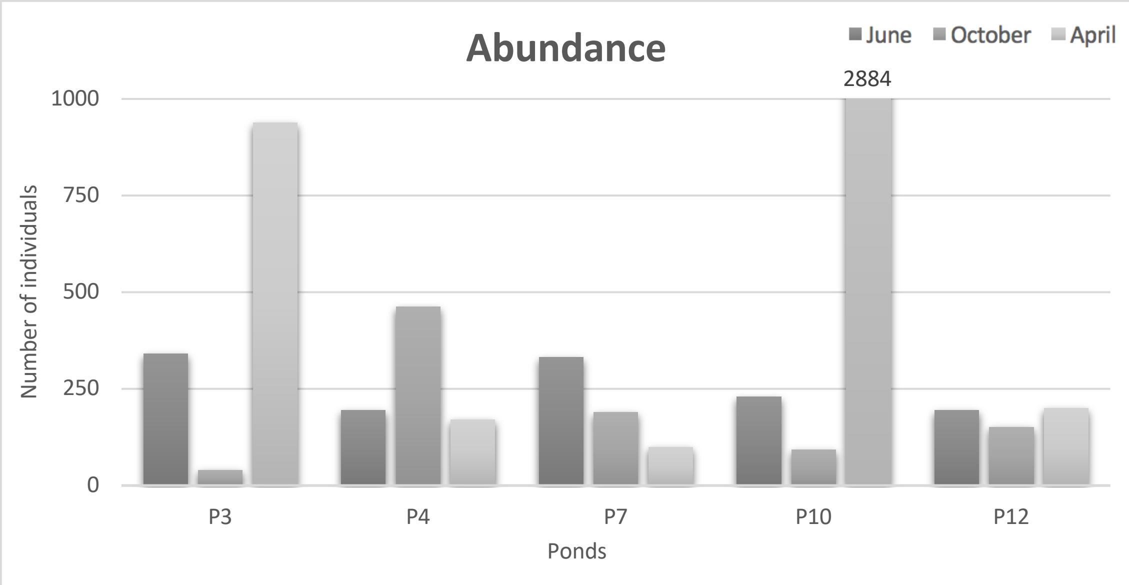

Figure 4 - Results of total abundance for the five ponds studied over the sampling period.

Figure 5 presents the diversity values for each pond in the three sampling periods. No significant differences along the seasonal gradient ($p = 0.104$) were recorded for diversity values. The greatest diversity value occurs in pond 12 in April and the lowest value was in pond 10 in October. However, diversity is generally low in all ponds over the sampling periods.

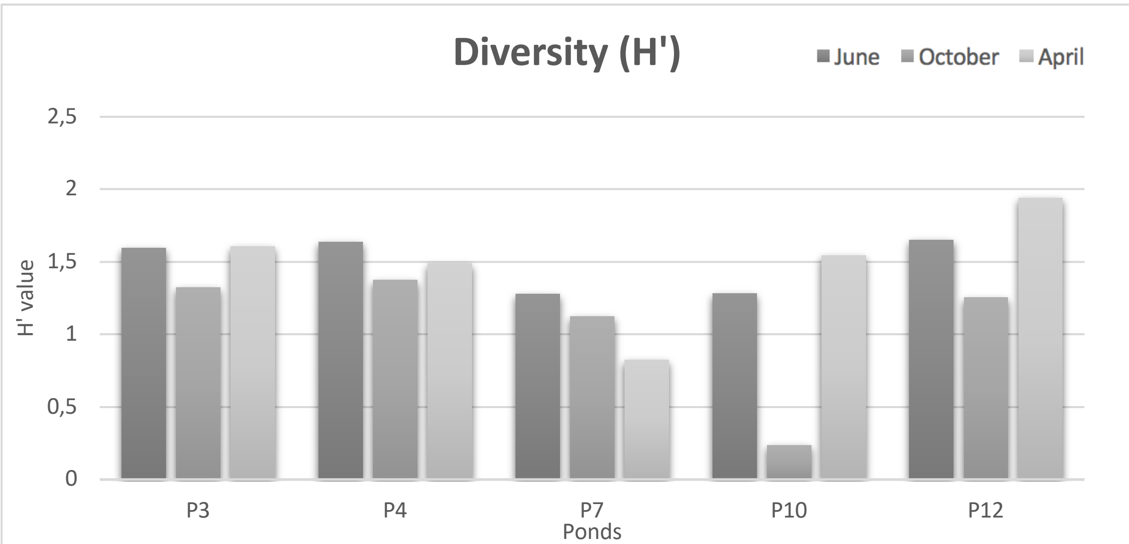

Figure 5 - Results of Shannon-Weaver (H') diversity index values calculated for the five ponds studied over the sampling period.

Taxa richness (Figure 6) seems to follow the diversity patterns, with the lowest value observed in pond 10 on October (only four families observed). The highest richness value occurred in the same pond (10) but in April. There is a noticeable variation in pond 3 and 10, with lower richness values recorded in October sampling. This variation is marked also for abundance and diversity values. It is also worth mention the pattern seen in pond 7, with a decrease alongside the seasonal gradient, that is also equal for richness, abundance and diversity values. However, no significant differences along the seasonal gradient (p = 0.072) were recorded for richness values.

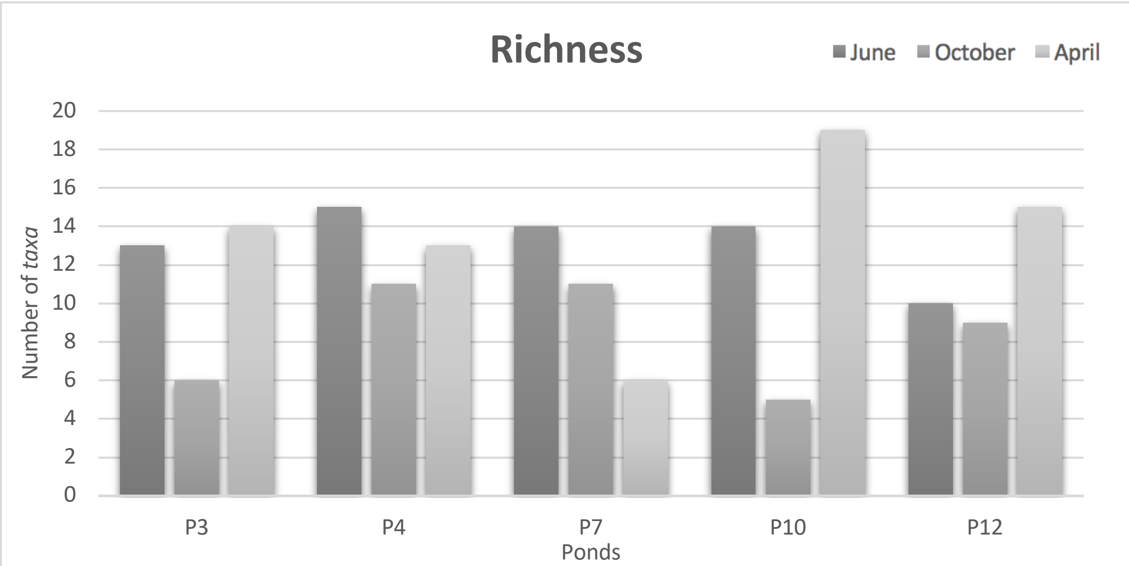

Figure 6 - Results of richness values for the five ponds studied in the sampling period.

Figure 7 shows the communities evenness. Overall no greater variation in data was observed and no significant differences along the seasonal gradient (p = 0.421) were recorded for evenness values. However, pond 10 in October seems to be an exception, with a decrease of evenness relatively to the other sampling periods and the other ponds. This low value showed that this pond had a dominant *taxon* in its macroinvertebrates community (Oligochaeta, n=89, *taxa* abundance = 93). Low richness values are also present on October in pond 3 (with oligochaetes and *Hygrotus* as dominant *taxa*) and on April in pond 7 (with Chironomidae as the dominant *taxon*).

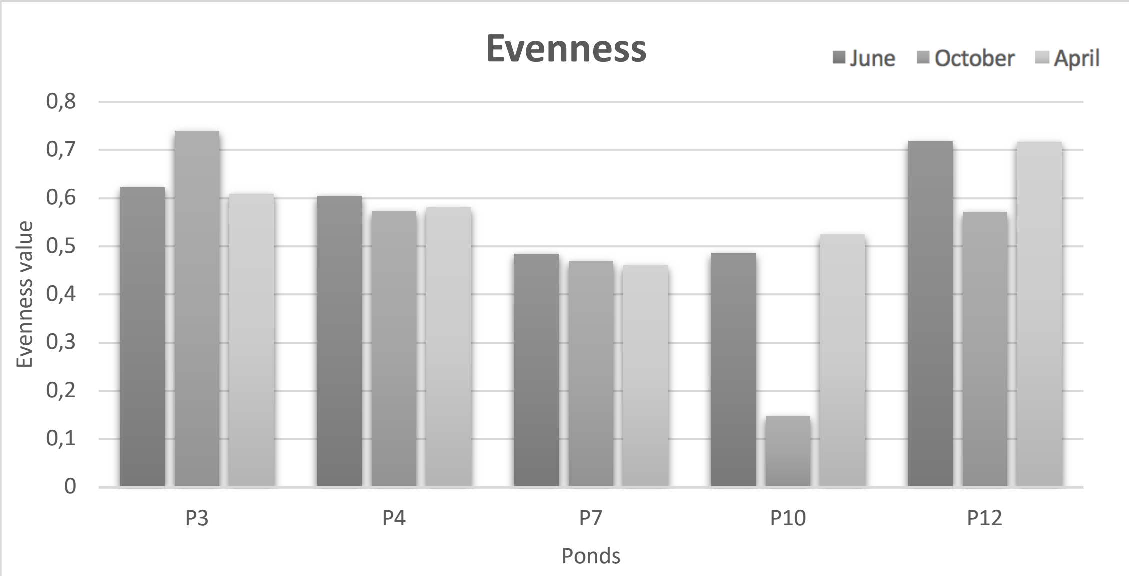

Figure 7 - Results of evenness values for the different sampled ponds for the different sampling periods.

Spearman's correlations tests were also performed to assess relations between water physical and chemical parameters and the communities' indexes. The calculated correlations show a significant ($p < 0.05$) and positive relations between abundance and pH (0.588); diversity (H') and pH (0.528) and O_2 % (0.528); *taxa* richness and temperature (0.567). Significant negative relations were also found between nitrates and diversity (-0.581) and *taxa* richness (-0.548).

A Correspondence Analysis (CA) was also performed in order to evaluate invertebrate distribution patterns across ponds and seasons (Figure 8). The acronyms used to represent each taxonomical group are presented in Appendix I – Table 6. Component 1 explains 25.47% (eigenvalue = 0.58) of species distribution variance while component 2 explains 15.76% (eigenvalue = 0.36). The CA analysis shows two distinguishable groups. A first group A (solid circle) linked to pond 12 is mainly formed by aquatic beetles (*Hydraena*, *Hygrotus*, *Acilius*) and damselflies (*Libellula*, *Coenagrion*), predators with good dispersion abilities and the latter a good bio-indicator of aquatic ecosystem quality. Group B (dotted circle) is linked to pond 4 with macroinvertebrate communities mainly constituted by caddisflies (*Athripsodes*, *Mystacides*) and mayflies (*Habrophlebia*), herbivores or detritivores and important leaf shredders having a great impact on primary production. These *taxa* are typical of waters in good ecological condition.

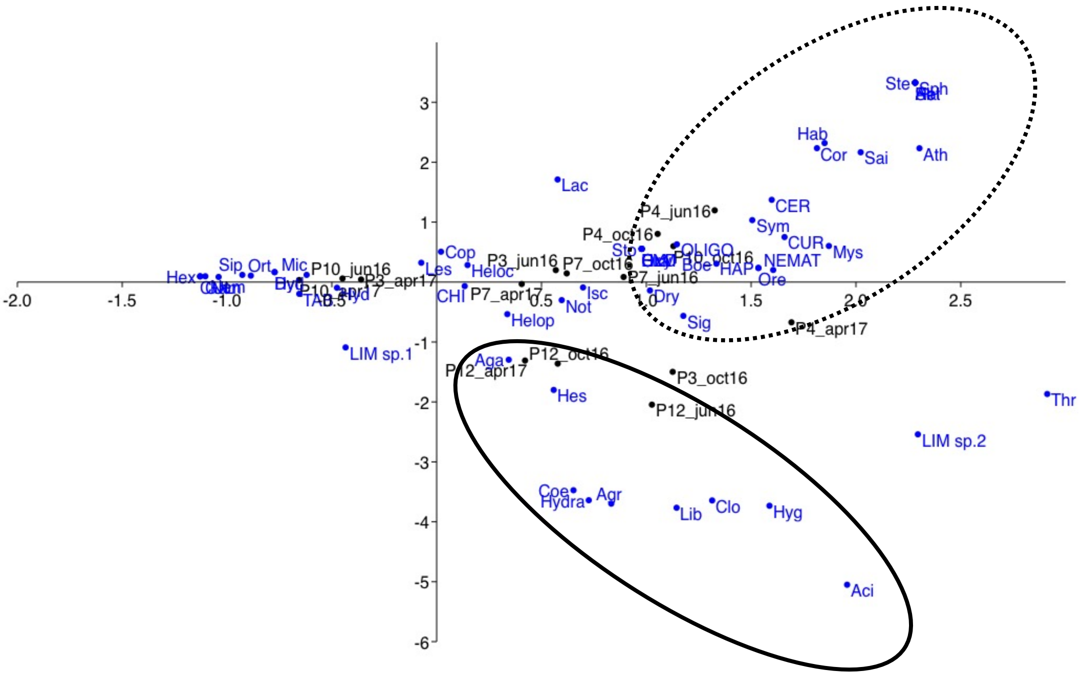

Figure 8 - Correspondence Analysis (CA) of the distribution of aquatic invertebrates through ponds and seasons.

IBMWP and IASPT indexes were also calculated and the results are presented in the Figures 9 and 10. There was no significant difference (p = 0.376) in IASPT values in the different sampling periods for all the ponds. On the other hand, a significant difference in IBMWP values (p=0.035) across the months was observed, namely between June and October (p = 0.018). In general, IBMWP values are lower in October (except for pond 7) relatively to other sampling periods. This result could imply that water quality in these months was lower. However, a closer look at the data points towards a relation with families' richness, thus implying that these low values are not due to the lack of water quality but to a lack of families present in the samples. This situation is corroborated by IASPT values (Figure 9), that show that the average score of the families in this sampling period has no great difference when compared to the other periods. The same situation is observed in April for pond 10, where IBWMP value was higher but IASPT value didn't follow this rise (Figure 8 and 9). This imply that the families score was similar but there were more families present in the sample (what is corroborated by abundance and richness values in this pond in the same period).

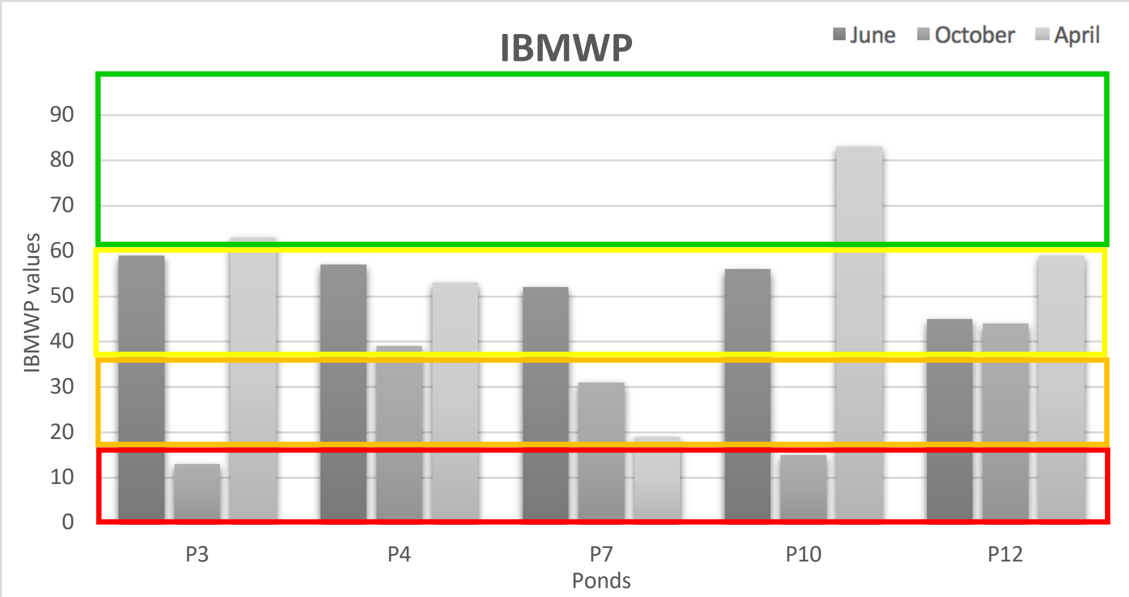

Figure 9 - Results of IBMWP index values for each pond over the sampling period. Each colour represents an ecological condition according to IBMWP scores in which red is bad (<15), orange is poor (16-35), yellow is moderate (36-60) and green is good (61-100).

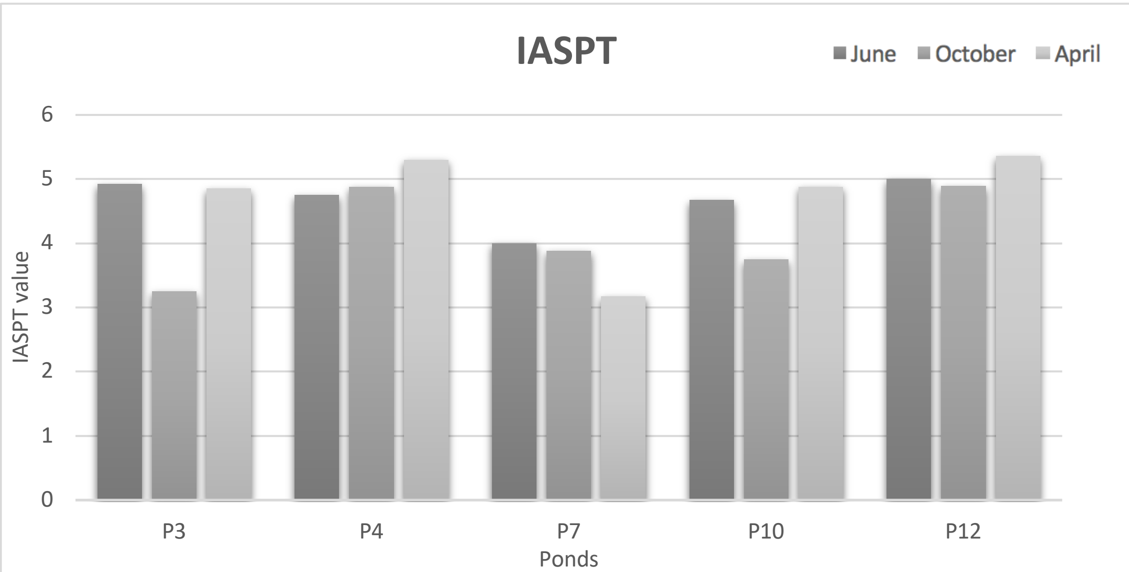

Figure 10 - Results of IASPT index values for each pond over the sampling period.

Ecological Quality Ratio was also calculated for each pond in the three sampling periods and the results are shown in Table 5. EQR values are associated with a categorical classification of the ecological condition of the waterbody. In this case, due to the lack of reference values for Portuguese natural ponds, mountain rivers of the north (type M, see INAG 2008) values were used once they were the better fit for the analysis of the pond data obtained. RQE value are positively correlated with all the other communities' indexes calculated and presented also positively correlations with pH and O_2 (%) and negative correlations with nitrates and ammonia.

Table 5 – $IPtI_N$ and ecological quality classification based on EQR values in comparison with type M rivers reference values (see INAG 2009, Appendix A).

Ponds	Months	$IPtI_N$	EQR	Ecological Condition for comparison with Mountain rivers of the north
P3	**June**	0.5536	0.5649	Fair
	October	0.2178	0.2223	Poor
	April	0.5896	0.6016	Good
P4	**June**	0.4784	0.4882	Fair
	October	0.3965	0.3965	Fair
	April	0.6491	0.6623	Good
P7	**June**	0.4518	0.4610	Fair
	October	0.2804	0.2804	Poor
	April	0.2100	0.2143	Poor
P10	**June**	0.4658	0.4753	Fair
	October	0.1763	0.1799	Bad
	April	0.6660	0.6796	Good
P12	**June**	0.5875	0.5994	Fair
	October	0.3884	0.3963	Poor
	April	0.6715	0.6852	Good

EQR values are also different in the different sample periods. In June all EQR values fit in the “Fair” category, but this condition is not observed in October, with a “Poor” condition for ponds 3, 7 and 12, only being “Fair” in pond 4 and reaching a “Bad” condition in pond 10, the pond located in the south of the road that receives water runoff directly from it. April is the month with the highest EQR values, with all ponds presenting a “Good” condition except for pond 7 that has a “Poor” condition. All values seem to be low, however the reference values are adapted to North Portugal mountain rivers where environmental constrains are not as severe as in alpine ponds.

Discussion

The five ponds studied belong to a specific alpine zone and thus present similar physical and chemical parameters as other alpine lakes and ponds in Europe (Füreder et al. 2006; Hinden et al. 2005; Tolotti et al. 2006) and in the Iberian Peninsula (Boavida & Gliwicz 1996; Toro et al. 2006). Local pond conditions are the main factors responsible for communities structures (Hill et al. 2017) hence it is crucial to analyse physical and chemical water parameters to understand communities' dynamics in a waterbody.

Sampling classification was made in accordance with the Principal Component Analysis (Figure 3) and three different groups were identified. Although all nitrogen compounds are overall low, there is a marked difference in nitrogen values across the seasons. This seasonal variation is noticeable in the groups formed in the PCA. Group 1 is formed by all October samples that had higher values of nitrates and ammonia concentration. Group 2 has the June samples and there seems to be a tendency towards higher pH water values. Temperatures also changed across the seasons, being the higher temperatures recorded in this month. In fact, shifts in temperature are important in alpine ecosystems because they serve as cues that can stimulate organisms to initiate or end certain processes that allow them to survive and prosper in alpine ponds. (e.g. pupation and emergence in the spring and supercooling processes in the winter) (Lencioni 2004). Lastly, group 3 had all spring samples. In this period after the snowmelt, there is a marked increase in oxygen values. Coloured Dissolved Organic Carbon also seems to be influencing the formation of group 3 although these values are only higher in April for ponds 3, 10 and 12. The rise in temperatures and the snowmelt allow a great macrophyte development in the spring. This is important in the ponds studied once they have high amounts of aquatic plants (mostly *Ranunculus ololeucus* J. Lloyd). It was already described that alpine ponds with vascular plants detritus and with peatland soil type generally have relatively higher CDOC values (Wissinger et al. 2016), what may explain the higher CDOC values found in these three ponds in April.

Conductivity and chlorophyll *a* content are other important parameters although not having a major role in the group formation in the PCA. Conductivity and TDS values are low through all the ponds and inside the range of values registered for other alpine ponds and lakes in Europe (Füreder et al. 2006; Hinden et al. 2005; Oertli et al. 2008; Rodrigues et al. 2010; Toro et al. 2006). However, pond 10 presents much higher values of conductivity when in comparison with the other four ponds studied. Differences in conductivity in other waterbodies in Serra da Estrela were already recorded (Rodrigues et al. 2010). In the work mentioned, the authors argue that changes in water conductivity are mainly caused by the use of salt for road deicing. The results obtained in the present study are in concordance with the conclusions withdrawn by Rodrigues et al. (2010) since the only pond that presented noticeable higher values of conductivity throughout all sampling periods was pond 10, a pond south of the road that receives water from surface runoff directly from the road. However, in this study, it was not possible to withdraw conclusions of changes in macroinvertebrates communities' dynamics related to the high conductivity values portrayed in pond 10.

Chlorophyll *a* varies from 0 to nearly 6 µg/L what points towards an oligotrophic to mesotrophic state according to the Trophic State Index (Carlson 1977). Chlorophyll *a* concentration was very low in June and April in pond 4 and in April in pond 12, not being detected in the chemical analysis. However, higher values appear for pond 3 in April (3.204 µg/L), in pond 7 on October (5.547 µg/L) and in pond 10 in April (4.272 µg/L), values normally attributed to mesotrophic lakes according to Carlson (1977). These two higher values are over the range of reference condition values (2.7 – 3.3 µg/L) attributed to mid-altitude shallow lakes proposed by Poikane et al. (2010) and the chlorophyll *a* content in pond 7 on October even passes the range accepted as the boundary to good quality (3.6 – 4.4 µg/L) proposed by the same authors. However, the reference values proposed by Poikane et al. (2010) are not designed for alpine ponds. Moreover, even higher values of chlorophyll *a* were already measured in alpine glacial ponds with granitic bedrock surroundings in the Tatra Mountains (Hamerlík et al. 2014). It is also worth mentioning that Hamerlík et al. (2014) compared alpine lakes and ponds and recorded significantly higher values of chlorophyll *a* content for ponds than for lakes in the same region. Thus, it is necessary to establish new reference values for high altitude lakes and ponds.

Regarding the macroinvertebrates communities' data, a great variation in abundances in pond 3 and pond 10 were recorded (Figure 4), with low values in October and extremely high values in April. Oxygen parameters (% and mg/L), that are correlated with macroinvertebrates abundance, diversity and taxonomic richness, were highest in these two ponds in the April sampling. The influence of oxygen in organisms abundance was already described in another study in a temporary pond in Italy, where habitats with lower dissolved oxygen presented reduced macroinvertebrates abundance values and lower faunal diversification (Bazzanti et al. 2010). In addition, both ponds mentioned have similar hydromorphologic characteristics, being rich in aquatic plants that vanish during the winter and grow back again in the beginning of the favourable season. This changes in aquatic vegetation biomass have an important impact on macroinvertebrates abundance and diversity as already shown for lakes (Schramm & Jirka 1989) and ponds (Declerck et al. 2011; Matthew J. Hill et al. 2017), what is not seen in the other studied ponds that are not rich in vegetation. Pond 12 is the most stable when referring to macroinvertebrates abundancy. However, in this pond were observed individuals of *Pelophylax perezi* in all sampling periods, what may be the major factor controlling this pond food web due to the strength of top-down control in shallow ponds as already described by Jeppesen et al. (1997).

Overall diversity (H') values are low in the studied ponds (Figure 5). This observation was expected since the local diversity of alpine ponds seems to be low, as already shown by Hamerlík et al. (2014) in a study that surveyed 25 ponds and 34 lakes in the Tatra Mountains. Although being low, Serra da Estrela alpine ponds' aquatic macroinvertebrate diversity is similar to other ponds and lakes in Europe (Füreder et al. 2006). However, the low diversity of each pond is in part compensated by generally high among-site diversity, since different ponds can provide different habitats, and this habitat heterogeneity is associated with a wider range of communities. Another factor that can explain the low diversity values is the constraints imposed by the high altitude that limits the colonization by some macroinvertebrate *taxa* (Hinden et al. 2005). Pond 10 had an extremely low diversity value in October. In the same pond and month, the highest nitrate concentration value was recorded. It was found a negative correlation between nitrates values and macroinvertebrate communities' diversity and taxonomic richness in the present study data. The impact of nitrogen enrichment was already studied for phytoplankton in alpine lakes and it was shown that nitrogen deposition may potentiate phytoplankton growth but is also responsible for shifts in the phytoplanktonic community composition towards larger and less palatable species that are considered as of poor food quality (Lafrancois et al. 2004; Nydick et al. 2004). Having these changes in consideration, the overall higher values of nitrates on October may be causing shifts in the phytoplankton's communities, what may cause an impact on zooplankton and macroinvertebrates communities, thus influencing the macroinvertebrates diversity and richness recorded. In fact, the phytoplanktonic communities of the alpine ponds mentioned in this work was already studied and shifts in the phytoplanktonic community of pond 10 were recorded for the same month in 2015, when a community dominated by Dinophyceae in September shifted to a community dominated by cyanobacteria in October (Moutinho 2016). Cyanobacteria are considered a poor quality food and its metabolites can be toxic to zooplankton, in a consequent negative impact on zooplankton fitness (Ger et al. 2016). This changes in zooplankton can cause an impact in the next trophic level, what may help explain the low diversity and richness of macroinvertebrates found in this pond in October. It is also worth mentioning that this pond (10) is located south of the road (Figure 1) and received water from surface runoff directly from the road above. Besides that, this pond is rich in aquatic plants and is surrounded by ground vegetation, providing optimal conditions for shepherds to feed their cattle, what was visible due to a high amount of faeces seen in the pond and the surrounding area. Animal waste is rich in nutrients such as nitrogen, and animal waste input in a waterbody is a concern when referring to water quality (Hubbard et al. 2004). This type of practices since the snowmelt to the end of the favourable season may accumulate this nutrient in the soil until the first autumn rains that will promote the nutrients runoff to the pond. The runoff phenomenon associated with nitrogen input by faeces direct deposition may be contributing to the higher nitrate values found in pond 10 on October.

Taxa richness follows the patterns of diversity, with October being the month with generally lower richness. Taxonomic richness is also positively correlated with oxygen values and a negative correlation with nitrates. Richness values found in the five ponds vary from 5 to 19, with an average value of 13 *taxa* per pond. Similar values were found in other alpine lakes and ponds across Europe (Füreder et al. 2006; Hinden et al. 2005; Oertli et al. 2008). A great area involving Switzerland, Italy, and Austria was studied with a macroinvertebrates survey in 55 lakes between 1840 and 2796 meters a. s. l. where the authors found that *taxa* richness was close to 16 *taxa* per lake when the lakes were at 2000 meters a. s. l. or less (Füreder et al. 2006). Although the values of the five studied ponds are a little lower than the above-mentioned lakes, a weak but important positive relationship between waterbody area and richness was already documented for natural ponds in Switzerland (Oertli et al. 2002). However, the same authors showed that a group of small ponds harbour more species than a single lake of the same total area, Showing the high conservation value of nets of small ponds in a landscape (Oertli et al. 2002). In Cirque of Macun National Park in Switzerland, high altitudinal ponds (>2600 meters) were surveyed and richness was on average 11.3 *taxa* per pond, with a minimum of 6 and a maximum of 24 (Oertli et al. 2008). Another study also in Switzerland studied 20 ponds in the alpine altitudinal belt across the Swiss Alps found a decrease in macroinvertebrates richness alongside an increase of altitude, with 9 *taxa* per pond on average at 1800 meters a. s. l. (Hinden et al. 2005), a similar altitude of the ponds depicted in this study. Close values were also observed in the Bogong High Plains, Australian Alps, in spring-fed alpine source pools at approximately 1800 a. s. l., where richness values vary from 5 to 12 *taxa* per pond (Clements et al. 2016). *Taxa* richness values obtained in this study are similar but slightly higher in comparison with the studies pointed out that were made in ponds during the summer. Although no significant difference in richness values across the seasons was found (p = 0.072), the *taxa* richness obtained seems higher in April when in comparison with the other sampling months. So, for conservational or research proposes, the seasonal variations should be taken into account, keeping in mind the higher probability of recording more different *taxa* in the spring, after the snowmelt, when the favourable season had already started. Evenness in the ponds studied seem to be similar across the seasons, with a lower value recorded in pond 10 in October. Although no significant correlation was detected between evenness and water physical and chemical parameters, a positive correlation between evenness and diversity was observed, what may thus explain why evenness in October in pond 10 is so low. Looking at the dataset, the sample of pond 10 in October was mainly constituted by the resistant Oligochaeta specimens, while other more sensitive *taxa* were absent during this sampling period. However, almost all values are high (approximately 0.5 or more) what points toward an equally distributed number of macroinvertebrates *taxa* in each pond's community.

The Correspondence Analysis (figure 7) allowed to separate two distinct groups, one linked to pond 12 (group A) and another linked to pond 4 (group B). Group A is mainly constituted by aquatic beetles (*Hydraena* - Hydrenidae, *Hygrotus*, *Acilius* - Dytiscidae), and damselflies (*Libellula* - Libellulidae, *Coenagrion* - Coenagrionidae). This pond presents the highest evenness values and have consistently higher IASPT values in comparison to the other samples, with little variance across the months analysed. This may indicate a stable ecological condition across the seasons marked by *taxa* related to high quality environments. Group B is mainly constituted by the epibenthic caddisflies larvae (*Athripsodes*, *Mystacides* - Leptoceridae) and by mayflies (*Habrophlebia* - Leptophlebiidae). Leptoceridae larvae are generally shredders or scrappers with a generalistic diet (Tachet et al. 1994). Furthermore, mayflies (*Habrophlebia* - Leptophlebiidae) are represented too, a taxonomic group that occupies diverse habitats and which larvae are detritivores (Tachet 2000). Group B is connected to pond 4, an isolated pond at the northern extremity of the study area and that is all surrounded by rocks and with a great marginal area with sand as subtract. The difficulties imposed to dispersion by the isolation of this pond may be one major factor contributing to the differentiation of the aquatic macroinvertebrates community in this pond. The different characteristics of each pond and the isolation allow the existence of different communities. The macroinvertebrates community differentiation seen in these two ponds shows that close but different ponds may harbour different species, what may help explaining why Oertli et al. (2002) detected that a set of small natural ponds have more species than a big pond or lake of the same total area. This information emphasizes the importance to include natural and alpine ponds in future conservation efforts.

Although IBMWP and IASPT are water quality indexes for lotic systems, more precisely for Iberian rivers, there is a lack of indexes that use benthic macroinvertebrates families as bioindicators for ecological quality of lentic systems. So, IBMWP and IASPT were used in the present work as a tool to obtain more information about the alpine ponds studied and thus the data obtained should be interpreted carefully. IBMWP values in the studied ponds varied from 13 to 83. The lowest values recorded were recorded in pond 3 and 10 in October, classifying these ponds as in bad ecological state. According to the same index, the water of pond 7 was classified as in a poor ecological state in October and April. Ponds 3 and 10 were in a good ecological state in April. The remaining ponds and sampling periods classified the waterbodies as in moderate ecological state. Analysing the values obtained, it is clear a significant difference between months. So, any future adaptation of this index to ponds and small shallow lakes, especially alpine ones, should take into account the sampling period (months). Another important note is that pond 3 and pond 10 presented the lowest (in October) and the highest (in April) values of the dataset. Both ponds mentioned are shallow wetlands with macrophytes (*Ranunculus ololeucus* Lloyd) as the main substrate. The abundance of areas with macrophytes as substrate may have a huge impact on the benthic macroinvertebrate communities. Declerck et al. (2011) shown that there is a positive correlation between macrophyte biomass and macroinvertebrates diversity in experimental ponds. Having this information into consideration, the type of habitats of a pond and the pond type itself (e.g. hydromorphological characterization, and local geology) should be taken into account for making conscientious ecological classifications of this kind of waterbodies. Taking IASPT values into account, there was little variance, with the minimum value at 3.17 in pond 7 on April and the maximum 5.36 in pond 12 in the same sampling period. When used alongside IBMWP, it is possible to see that although October presented low IBMWP values in comparison with the other two months sampled, this drop was not evident in IASPT values. Some caution may be taken when referring to the October sample period since IBMWP values in this period may point towards a lower water ecological quality. However, IBMWP is correlated with *taxa* richness (0.876). So, low values of IBMWP in October do not imply poor water ecological quality in that period, IBMWP values are low due to a lack of families present in the samples. An analysis of IASPT values allows to better understand this phenomenon, once there is no significant difference between in this index across the months sampled, what points towards a similar average score of the families in all sampled periods.

Ecological Quality Ratio was also calculated using macroinvertebrates families present in each sample. There was marked seasonal variation also with “Fair” condition in all ponds in June, with “Poor” to “Fair” in the October period and with mainly “Good” condition in April samples. EQR values are positively correlated with all communities' dynamics aspects analysed (abundance, diversity, richness, and evenness) and with the IBMWP and IASPT indexes, being that the same factors influencing the results for the indexes used in the present study are also modelling the EQR results obtained. Overall EQR values calculated are relatively low but that does not necessarily imply that the ponds present a poor or even fair ecological quality. High altitudinal ponds present great constrains as the temperature fluctuations, the short growth period and high snow-covered period or even strong UV radiation, among others (Hinden et al. 2005). These factors seem to be responsible to the limit of the colonization of the studied waterbodies, and this limitation may have a negative impact on the EQR values obtained. However, it is worth noting that these reference values are valid for Portuguese mountain rivers of the North (see INAG 2008), and there are no reference values for alpine ponds or even for natural ponds at lower altitudes, and the analysis of this data should be careful.

It is evident the lack of water quality reference values for high altitudinal alpine lakes and ponds in the literature and legislation. Therefore, future works should focus on establishing quality reference values for this type of waterbodies. Reference values would also allow a better monitoring of alpine waterbodies and would allow a detention of possible changes caused by climate change once these systems can be very sensible as already noted by Murphy et al. (2010).

Other problem associated with climate change is the possible impact of global warming on the faunal diversity of alpine ponds. In natural ponds, global warming is expected have an impact in species distributions with possible extinctions (Rosset & Oertli 2011). Temperature changes caused by global warming may reduce local diversity with the impoverishment of populations of cold stenothermal species. There is also the strong possibility of colonisation events by generalistic species. So, these events will lead to shifts in local communities composition (Rosset & Oertli 2011). Therefore, there is a necessity to document species present in alpine ponds in order to form suitable monitoring and conservational programmes.

In the Iberian Peninsula, new and complementary IBMWP and IASPT reference values should be established, in order to use these indexes in natural and alpine ponds, with take in account the constraints of these ecosystems. A similar index to the $IPtI_N$ (Índice Português de Invertebrados Norte – Northern Invertebrates Portuguese Index) should be created for alpine ponds and other natural ponds at different altitudes.

Referring to the studied ponds, further researches with emphasis in other faunal and floral groups should be carried out. On the other hand, the results of this work do not allow to discriminate effects of the use of salt in road deicing processes in the macroinvertebrates communities. Further studies on phytoplanktonic and zooplanktonic communities' seasonal dynamics in alpine ponds could be helpful to detect changes in the biota caused by water chemistry variations and their possible impacts on the food web.

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Appendix I – Macroinvertebrates recorded

Table 6 - Macroinvertebrates recorded. All specimens were identified to the lowest taxonomic group possible.

Taxa	Abrev.	Pond 3			Pond 4			Pond 7			Pond 10			Pond 12		
		Jul/16	Oct/16	Apr/17	Jul/16	Oct/16	Apr/17	Jul/16	Oct/16	Apr/17	Jul/16	Oct/16	Apr/17	Jul/16	Oct/16	Apr/17
Nematoda	NEMAT	0	1	0	0	2	0	3	1	0	0	0	0	0	0	0
Oligochaeta	OLIGO	126	14	86	45	208	57	183	77	23	28	89	84	0	7	21
Chironomidae	CHI	112	0	304	99	132	4	102	95	71	26	0	626	77	86	53
Ceratopogonidae	CER	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Elmidae	ELM	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroporidae	HYD	29	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Curculionidae	CUR	0	0	0	1	0	0	5	0	0	0	0	0	0	0	0
Tabanidae	TAB	0	1	7	0	0	0	0	0	0	0	0	6	0	0	0
Haplotaxidae	HAP	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Culicidae	CUL	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Other Genus * Limnephilidae (1)	LIM sp.1	0	0	31	0	0	1	0	0	0	3	0	97	0	0	61
Other Genus * Limnephilidae (2)	LIM sp.2	12	0	0	0	0	73	8	0	0	1	0	0	35	0	1
Siphlonurus	*Sip*	42	0	345	0	0	0	0	0	0	150	0	1037	0	0	0
Graphoderus	*Gra*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dryops	*Dry*	2	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Lestes	*Les*	3	0	0	0	0	0	0	0	0	3	0	1	0	0	0
Oxygastra curtisii	*Oxy*	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Stomatochlora	*Sto*	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Allogamus	*All*	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
Athripsodes	*Ath*	0	0	0	93	14	20	0	0	0	0	0	0	1	1	1
Mystacides	*Mys*	0	0	0	2	9	3	0	0	0	0	0	0	0	0	3
Setodes	*Set*	0	0	0	23	0	0	0	0	0	0	0	0	0	0	0
Habrophlebia	*Hab*	0	0	0	7	83	0	0	0	0	0	0	0	0	0	0
Sialis fulginosa	*Sai*	0	0	0	3	8	1	0	0	0	0	0	0	0	0	0
Haliplus	*Hal*	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Laccophilus hyalinus	*Lac*	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Stenelmis	*Ste*	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Sphaerium	*Sph*	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Libellula	*Lib*	0	0	0	0	0	0	7	3	0	3	0	1	30	40	17
Boeria irene	*Boe*	0	0	0	0	0	0	4	3	0	0	0	0	0	0	0
Hygrotus	*Hyg*	0	16	0	0	0	0	9	0	0	0	0	0	15	3	7
Sigara	*Sig*	0	0	1	0	1	0	6	2	0	0	1	0	0	1	3
Notonecta	*Not*	0	0	0	0	0	0	1	5	0	2	0	1	1	0	0
Orectochilus	*Ore*	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Hydroglyphus	*Hyd*	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Cloeon dipterum	*Clo*	1	0	0	4	1	1	1	0	3	1	0	0	28	7	21
Dytiscus	*Dyt*	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Hydroporus	*Hyd*	0	0	16	0	0	1	0	0	1	5	0	3	0	0	1
Helochares	*Heloc*	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
Helophorus	*Helop*	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
Coenagrion	*Coe*	0	0	0	0	0	0	0	0	0	0	0	2	3	0	3
Acilius sulcatus	*Aci*	0	0	0	0	0	1	0	0	0	0	0	0	5	0	0

Agabus	*Aga*	0	8	8	0	0	0	0	1	0	0	1	5	0	0	0
Corbicula fluminea	*Cor*	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Hesperocorixa	*Hes*	0	0	0	0	0	0	0	1	1	0	0	1	0	2	1
Sympetrum	*Sym*	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
Copelatus	*Cop*	0	0	3	0	0	0	0	0	0	0	1	0	0	0	0
Agrypnia varia	*Agr*	0	0	0	0	0	0	0	0	0	0	0	0	0	4	6
Nemoura	*Nem*	0	0	109	0	0	6	0	0	0	0	0	787	0	0	0
Microvelia	*Mic*	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Orthetrum	*Ort*	0	0	10	0	0	0	0	0	0	0	0	11	0	0	0
Gammarus	*Gam*	0	0	11	0	0	0	0	0	0	0	0	212	0	0	0
Thraulus bellus	*Thr*	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Ischnura	*Isc*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Hexatoma obscura	*Hex*	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Hydraena	*Hydra*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

* LIM sp.1 and LIM sp.2 were used when it was not possible to identify the genus within Limnephilidae family, but separation into two different groups was possible.

Appendix II – IBMWP and IASPT indexes

IBMWP index: ∑ of the scores of each family present in a sample

IASPT index: $\frac{IBMWP}{n}$, n = the total number of families in a sample

Table 7 - Families scores used in IBMWP and IASPT indexes.

EP: Siphlonuridae, Heptageniidae, Leptophlebiidae, Potamanthidae, Ephemeridae	10
PL: Taeniopterygidae, Leuctridae, Capniidae, Perlodidae, Perlidae, Chloroperlidae	10
TR: Phryganeidae, Molannidae, Beraeidae, Odontoceridae, Leptoceridae, Goeridae	10
TR: Lepidostomatidae, Brachycentridae, Sericostomatidae	10
DI: Athericidae, Blephariceridae	10
HE: Aphelocheiridae	10
OD: Lestidae, Calopterygidae, Gomphidae, Cordulegasteridae, Aeshnidae	8
OD: Corduliidae, Libellulidae	8
TR: Psychomyiidae, Philopotamidae, Glossosomatidae	8
CR: Astacidae	8
EP: Ephemerellidae, Prosopistomatidae	7
PL: Nemouridae	7
TR: Rhyacophilidae, Polycentropodidae, Limnephilidae, Ecnomidae	7
MO: Neritidae, Viviparidae, Ancylidae, Thiaridae, Unionidae	6
TR: Hydroptilidae	6
CR: Gammaridae, Atyidae, Corophiidae	6
OD: Platycnemidae, Coenagrionidae	6
EP: Oligoneuriidae, Polymitarcidae	5
CO: Dryopidae, Elmidae, Helophoridae, Hydrochidae, Hydraenidae, Clambidae	5
TR: Hydropsychidae	5
DI: Tipulidae, Simuliidae	5
PT: Planariidae, Dugesiidae, Dendrocoelidae	5
EP: Baetidae, Caenidae	4
CO: Haliplidae, Curculionidae, Chrysomelidae	4
DI: Tabanidae, Stratiomyidae, Empididae, Dolichopodidae, Dixidae	4
DI: Ceratopogonidae, Anthomyidae, Limonidae, Psychodidae, Sciomyzidae, Rhagionidae	4
NE: Sialidae	4
AN: Piscicolidae	4
AC: Hydracarina	4
HE: Mesoveliidae, Veliidae, Hydrometridae, Gerridae, Nepidae, Naucoridae, Pleidae	3
HE: Notonectidae, Corixidae	3
CO: Helodidae, Hydrophilidae, Hygrobiidae, Dytiscidae, Gyrinidae	3
MO: Valvatidae, Hydrobiidae, Lymnaeidae, Physidae, Planorbidae	3
MO: Bithyniidae, Bythinellidae, Sphaeriidae	3
AN: Glossiphoniidae, Hirudidae, Erpobdellidae	3
CR: Asellidae, Ostracoda	3
DI: Chironomidae, Culicidae, Thaumaleidae, Ephydridae	2
AN: Oligochaeta	1
DI: Syrphidae	1

EP – Ephemeroptera; **PL** – Plecoptera; **TR** – Trichoptera; **DI** – Diptera;

HE – Heteroptera; **MO** – Mollusca; **AN** – Annelida; **CR** – Crustacea; **CO** – Coleoptera;

OD – Odonata; **NE** – Neuroptera; **AC** – Acarina; **PT** – Plathelminthes.

Appendix III – Ponds photographs

Figure 11 - Pond 3 (June of 2016).

Figure 12 - Pond 4 (June of 2016).

Figure 13 - Pond 7 (June 2016).

Figure 14 - Pond 10 (June 2016).

Figure 15 - Pond 12 (June 2016).